Autonomic microganglion cells: a source of acetylcholine in the rat carotid body

Estelle B. Gauda,1 Reed Cooper,1 Sheré M. Johnson,2 Gabrielle L. McLemore,3 and Cathleen Marshall1
1Division of Neonatology, Department of Pediatrics, Johns Hopkins Medical Institutions, Baltimore, Maryland 21287-3200; 2Department of Physiology and Biophysics, College of Medicine, Howard University, Washington, DC 20059; and 3Department of Biology, School of Computer, Mathematical and Natural Sciences, Morgan State University, Baltimore, Maryland 21251

Submitted 22 August 2003; accepted in final form 17 September 2003

Oxygen Sensing in Health and Disease

PERIPHERAL ARTERIAL CHEMORECEPTORS in the carotid body (CB) play a critical role in modulating ventilation by uniquely sensing changes in \( \text{O}_2 \) tension (17). Peripheral arterial chemoreceptors in the CB consist of three major neuronal components that include 1) type I chemosensory cells, resembling presynaptic neurons, which contain neurotransmitters and autoreceptors; 2) type II cells, comparable to glia, which are not known to contain neurotransmitters; and 3) carotid sinus nerve fibers, innervating type I cells, which have soma in the petrosal ganglion and contain receptors for different neurochemicals.

In the healthy fetus, peripheral arterial chemoreceptors do not significantly contribute to fetal breathing, nor is their activity necessary for establishing rhythmic breathing at birth (24) irrespective of the level of hypoxia, which is ~25 Torr. However, multiple studies (5, 8, 20), in numerous mammalian models, support a role for peripheral arterial chemoreceptors in contributing to stable ventilation at a critical period of development, during early postnatal life, which establishes rhythmogenesis that is sustained throughout life. After the resetting of the peripheral arterial chemoreceptor to a higher \( \text{O}_2 \) tension, after birth there is a gradual increase in hypoxic chemosensitivity of these chemoreceptors, with early postnatal maturation, in essentially all mammalian species (1, 4, 25). Asphyxial apnea associated with upper airway obstruction is thought to occur in infants at risk for sudden infant death. Activation of peripheral arterial chemoreceptors decreases the time to arousal and reestablishes airway patenty in immature and mature models during asphyxial apnea. Thus understanding of cellular mechanisms involved in maturation of peripheral arterial chemoreceptor function is key.

To date, it is not known how \( \text{O}_2 \) tension is sensed by type I cells. It is, however, well established that hypoxia causes type I cells to depolarize and release neurotransmitters, which bind autoreceptors expressed by type I cells or heteroreceptors on nerve terminals innervating type I cells (16). The predominant excitatory transmitter synthesized and released by type I cells, in response to hypoxia, is still a matter of debate (16). Earlier work led to the cholinergic hypothesis that acetylcholine (ACh) perhaps was the major excitatory neurotransmitter involved in hypoxic chemotransmission (9, 19). Subsequent studies either were unable to substantiate or even refute this hypothesis (7, 29, 34). Recent evidence, predominately from physiological and pharmacological studies performed in the adult cat (11) and immunohistochemical and electrophysiological studies in primary culture of rat type I cells (32), has brought about a resurgence of the excitatory role of ACh on peripheral chemoreceptor activation in response to hypoxic exposure (10, 12).
Choline acetyltransferase (ChAT) is the rate-limiting enzyme for the synthesis of ACh, and the vesicular ACh transporter (VACHT) transports ACh into synaptic vesicles. Expression of ChAT and VACHT mRNA and protein serve as definitive markers for cholinergic neurons in the periphery and in the central nervous system (CNS) (43). In this study, we hypothesized that ChAT and VACHT mRNA levels would increase with postnatal maturation and that hypoxia-induced upregulation of these mRNAs would be greatest in the more mature animals. Semiquantitative in situ hybridization histochemistry (ISHH) and immunohistochemistry (IH) were used to localize mRNAs and protein, respectively, to specific neurons and cells in peripheral arterial chemoreceptors. A preliminary report of this work has been presented and referred to in a recently published review article by Gauda (14).

METHODS

Neonatal rat pups born to time-dated Sprague-Dawley dams were used in experimental protocols approved by the Animal Care and Use Committee at Johns Hopkins University. To determine the effect of development on the pattern of gene expression for ChAT and VACHT mRNAs, tissues were removed from rat pups at postnatal days P2, P5, P11, and P25, respectively; n = 5 at each age) and processed for ISHH. The expression of ChAT and VACHT mRNAs was determined in P4 (n = 12) and P14 (n = 12) animals after a 4-h exposure to hypoxia (10% O2-balance N2; n = 6 each age) or air (21% O2-balance N2; n = 6 each age) in a Plexiglas chamber. This level and duration of hypoxic exposure have been shown to increase tyrosine hydroxylase (TH) gene expression in the rat CB (6). After exposure, animals were briefly anesthetized with halothane and decapitated before removal of tissues. The bifurcation of the carotid artery, including the CB, nodose-petrosal-jugular ganglion (NG-PG-JG) complex and the superior cervical ganglion (SCG) were quickly removed en bloc, placed in embedding media (Triangle Biomedical Sciences, Durham, NC), quick frozen on dry ice, and stored at −70°C until further processing for ISHH.

ISHH. Tissue blocks were cut in 12-μm sections on a cryostat and thaw-mounted onto gelatin-chrome alum-subbed slides. Slides were fixed in 4% paraformaldehyde, acetylated in fresh 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated in an ascending series of alcohols, delipidated in chloroform, and rehydrated in a descending series of alcohols. Slides were air dried and then stored at −20°C.

Because multiple splice variants exist for ChAT and VACHT, we used clones that contained the entire coding region of the rat ChAT (23) and VACHT (2, 37) genes. (These clones were kindly provided by Dr. Hidemi Misawa, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan). The cDNAs were subcloned into Bluescript (Stratagene) plasmids to allow for in vitro transcription to generate antisense ribonucleotide probes. Specificity of the sense and antisense digoxigenin-labeled ribonucleotide probes has been well characterized (22, 23). In our experiments, probes were labeled with 35S-labeled UTP to increase the hybridization of the probe to detect very low levels of mRNAs, and ISHH was performed, as previously described (15). Serial sections were also hybridized with antisense ribonucleotide probe for TH, the rate-limiting enzyme in catecholamine synthesis, complementary to base pairs 1,120–1,488 of the rat cDNA (18). Before overnight hybridization at 35°C, sections were treated with proteinase A (2.5 μg/ml; Fisher Biotech, Fairlawn, NJ) for 5 min and rinsed with phosphate-buffered saline. As outlined in Gauda et al. (15), after hybridization, the slides were rinsed, treated with RNase A (20 mg/ml; Sigma Chemical, St. Louis, MO), washed at 60°C in ×0.2 saline-sodium citrate buffer (Sigma Chemical), rinsed in deionized water, and air dried. Slides were then dipped in Kodak photographic emulsion, dried, and exposed in the dark at −20°C for 8–10 wk. Postexposure slides were thawed, developed with Dektol (Eastman Kodak, Kodak, NY), fixed in Kodak Fixer, rinsed, and counterstained with thionin, and coverslips were applied with Permount.

To verify the specificity of the ribonucleotide probes by ISHH, rat coronal brain sections (Bregma 0.70, Paxinos and Watson) were hybridized, exposed, and developed simultaneously with the experimental slides. Control slides exhibited previously established patterns of expression for ChAT and VACHT mRNA, and protein in striato-neuronal neurons (21–23). In addition, the pattern of ChAT and VACHT gene expression differed in the CB, NG-PG-JG complex, and the SCG, further demonstrating probe specificity.

IH. Tissue sections were processed for VACHT and TH immunoreactivity (IR) alone or were sequentially processed for VACHT and then TH protein in the CB, NG-PG-JG complex, and the SCG. All tissue sections of the entire CB, NG-PG-JG complex, and the SCG from animals at 4, 8, 13–15, and 28 postnatal days (n = 3 at each age) were processed for VACHT IR alone or were double-labeled to detect VACHT and TH IR. Primary and secondary antibody combinations for VACHT protein visualization included 1) VACHT goat polyclonal IgG directed to the COOH-terminal of rat VACHT (1:100 dilution) and 2) mouse anti-goat conjugated to alkaline phosphatase (1:200 dilution). Both primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary and secondary antibody combination for TH protein visualization included 1) mouse anti-TH IgG2a monoclonal antibody (1:500 dilution; Chemicon International, Temecula, CA) and 2) biotinylated rat anti-mouse IgG2a (1:100; BD Biosciences Pharmigen, San Diego, CA). All antibodies were diluted in 0.3% Triton X-100 in Tris-buffered saline (TBS). All incubations with primary and secondary antibodies were performed in a humid Plexiglas container.

Single-labeling IH to detect VACHT. Tissue sections were cut, thaw mounted onto gelatin-chrome alum-subbed slides, fixed in 4% paraformaldehyde, and washed three times for 5 min each in 1 M TBS (pH 7.4). The slides were then incubated in ice-cold acetone for 10 min and subsequently rinsed three times with TBS. Nonspecific binding was blocked by incubation in 3% BSA containing 0.1% Triton X-100 in TBS for 30 min. Slides were incubated with the anti-VACHT antibody overnight at room temperature, washed with TBS, incubated with mouse anti-goat-alkaline phosphatase secondary antibody for 2 h at room temperature, washed with TBS, placed in visualization buffer containing 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, 0.34 mg/ml nitro blue tetrazolium, 0.18 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (4-toluidine salt), and 5 mM levamisole for 2 h. VACHT expression was visualized as a dark blue reaction product. For single-labeling IH, the reaction was stopped in water, and coverslips were applied with an aqueous based mounting media.

Double-labeling IH to detect VACHT and TH IR. Additional slides were processed for both VACHT and TH IR. After VACHT-IR visualization, the slides were washed three times in TBS, incubated in 3% hydrogen peroxide in TBS for 10 min, and incubated overnight with the primary antibody (anti-TH) at 4°C. Tissues were washed followed by incubation with biotinylated rat anti-mouse IgG2a secondary antibody at room temperature. After the slides were washed in TBS, they were incubated in freshly prepared streptavidin-horseradish peroxidase reaction mixture for 4 min followed by several washes in TBS. TH IR was visualized by incubating the slides in aminoethyl carbazole substrate (Zymed, San Francisco, CA) for 10 min. TH IR was visualized as a red reaction product. After visualization of the TH IR, the slides were rinsed with distilled water, and coverslips were applied with a water-soluble mounting media. Control slides were included and processed in the same manner as experimental slides except the primary antibodies were omitted or the antibody was preabsorbed with blocking peptide. VACHT antibody was preabsorbed with blocking peptide (Santa Cruz Biotechnolog) in a ratio of 1:100 (antibody to blocking peptide) overnight before being applied to tissue sections and further processed for IH.

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Data analysis. Qualitative and semiquantitative analysis of the data were performed. The pattern of expression for ChAT and VACHT mRNA, and IR for VACHT protein was determined in the CB, NG-JG-PG complex, and the SCG during the first 25 days of postnatal development. The effect of hypoxic exposure on ChAT and VACHT mRNA expression was determined by semiquantitative analysis of silver grains in animals exposed to air or hypoxic gas. Silver grains, generated by $^{35}$S in the emulsion, were analyzed by using a Nikon microscope and Macintosh image analysis program (National Institutes of Health Image). Darkfield images were captured and digitized at $\times 400$ and semiquantified by using silver grains representing VACHT mRNA expression in the NG-PG-JG complex from animals exposed to air or hypoxia at P4 and P14. The number of silver grains was obtained for 10 ganglion cells per section of the NG-PG-JG complex with five to seven tissue sections analyzed per animal. The mean number of silver grains per ganglion cell per animal was determined, and comparisons were made between P4 and P14 animals exposed to air or hypoxia by using the Student’s unpaired t-test with a Bonferroni correction for multiple comparisons. Thus significance was accepted at $P < 0.025$. Comparisons (qualitative and semiquantitative) were only made between data obtained from slides that were processed for ISH, hybridized, exposed, and developed together under the same conditions.

RESULTS

Constitutive and induced expression of VACHT and ChAT mRNAs in the NG-PG-JG complex and CB during postnatal development and exposure to hypoxia. Regional distribution profiles for VACHT and ChAT mRNAs were qualitatively the same for each age group; however, VACHT mRNA expression was uniformly greater than ChAT mRNA in all tissues examined. Constitutive expression of VACHT mRNA was detected in the NG-PG-JG complex in all animals, although the level of expression decreased with maturation (Fig. 1). VACHT mRNA levels were $31 \pm 1.4$ and $17 \pm 1.6$ (means $\pm$ SE, $P < 0.001$) silver grains/ganglion cell in animals at P4 and P14, respectively. Hypoxic exposure did not change the level of expression in the NG-PG-JG complex in either the 4- or 14-day-old animals (Fig. 2). VACHT mRNA expression was only seen in isolated cells on the periphery of the CB or within nerve fibers innervating the CB, as shown in Fig. 3, for animals at 2, 5, and 11 postnatal days. Hypoxic exposure did not induce ChAT or VACHT gene expression in the CB or change the pattern of expression in the other tissues.

Comparison of ChAT and VACHT mRNA expression profile with that of TH mRNA expression. Although ChAT and VACHT mRNA expression were not seen within the CB, this was not the case for TH mRNA expression. For example, a representative series of photomicrographs, showing the expression of ChAT, VACHT, and TH mRNAs on 12-μm serial sections of the CB and NG-PG-JG complex from one animal at P4 after 4 h of exposure to hypoxia, is shown in Fig. 4. Serial sections of the CB showed essentially undetectable levels of ChAT (Fig. 4A) and VACHT (Fig. 4B) mRNAs, whereas TH mRNA expression was abundantly expressed in the CB (Fig. 4C). Low levels of ChAT mRNA expression (slightly above background) were seen in a few cells, moderate levels of VACHT mRNA expression were seen in numerous cells, and high levels of TH mRNA expression were seen in a moderate number of cells on serial sections of the NG-PG-JG complex.

Pattern of VACHT-IR in the CB and SCG during postnatal development. The distribution of VACHT IR detected by IH was similar to that of the distribution of the mRNA in the tissues from animals between P4 and P28. Intense VACHT IR was detected in cells on the periphery of the CB, which are most likely autonomic microganglion cells (Fig. 5, A–D). On average, three to six microganglion cells per animal were seen within and on the periphery of the CB. This number was similar in animals at P4 and P14. Within the CB, the distribution of VACHT expression was linear and appeared to surround clusters of type I cell as exemplified in the photomicrograph from an animal at P15 (Fig. 5, E and F). Numerous cells, intensely positive for VACHT IR, were seen in larger nerves from animals at each postnatal age, as shown in Fig. 6. Cells intensely positive for VACHT were seen in nerve fibers next to the CB (Fig. 6, A and B) and large nerve fibers within the tissue bloc (Fig. 6, C and D). VACHT IR appeared as an intricate latticework surrounding the ganglion cells throughout the SCG (Fig. 6, A and C).

Comparison of the pattern of VACHT IR and TH IR in the SCG, NG-PG-JG complex, and CB by using double-labeling IM. Double-labeled IH was performed to determine whether VACHT and TH IRS are colocalized. Essentially all ganglion
cells in the SCG were intensely positive for TH IR, whereas preganglionic nerve fibers, innervating the SCG, were intensely positive for VACHT IR, (Fig. 7A). Ganglion cells in the NG-PG-JG complex were positive for VACHT IR; however, VACHT IR ganglion cells were larger than those ganglion cells that were positive for TH IR in the NG-PG-JG complex (Fig. 7B), and the two chromogens were not colocalized. As shown in Fig. 8, microganglion cells, in nerve fibers and on the periphery of the CB, were intensely positive for VACHT IR (Fig. 8, A and B), which is identical to the pattern of expression seen in the IH experiments that detected VACHT IR alone. VACHT IR was not colocd with TH IR within the CB; however, VACHT IR nerve fibers were closely juxtaposed to TH IR cells within the CB (Fig. 8, B and D), and microganglion cells were embedded within the CB (Fig. 8, C–F). Small clusters of microganglion cells were seen adjacent to clusters of TH IR cells, which are presumably clusters of type I cells (Fig. 8, E and F). Control slides, processed without the addition of primary antibody for TH and VACHT, were negative for IR, whereas control slides in which the antibody was preabsorbed with the antigen (1:100 antigen-to-antibody ratio) overnight before application to the slides showed faint immunostaining for VACHT.

DISCUSSION

Our findings indicate that cholinergic markers for ACh traits are localized within microganglion cells and nerve fibers innervating the rat CB. However, these results clearly show lack of coexpression of ACh traits with TH IR in the CB, NG-PG-JG complex, or SCG. Furthermore, contrary to our original hypothesis, postnatal development or hypoxic exposure did not induce ChAT or VACHT mRNA expression in the CB or change the level of mRNA expression in the NG-PG-JG complex even though the level and duration of hypoxic exposure has been shown to increase TH gene expression in the rat CB (6). Last, the results of this present study, using sensitive cholinergic markers for gene and protein expression, clearly demonstrate the presence of ACh traits in microganglion cells and nerve fibers, but not in type I cells in the rat CB, indicating that the source of ACh during the first 28 days of postnatal development is mostly microganglion cells and nerve fibers innervating the CB. Even though sensitive cholinergic markers (e.g., ribonucleotide probes that have the complementary sequence of the entire coding sequence of the rat gene) were used for detection of gene and protein expression and VACHT antibodies, they did not detect mRNA or IR, respectively, for these cholinergic markers in type I cells in the rat CB during the first 28 days of postnatal development. These findings are particularly relevant to the possible source of ACh in the CB and its role in hypoxic chemotransmission during mammalian development.

The results of the present study indicate that ACh found within the CB originates mainly from nerve fibers and autonomic microganglion cells embedded within the CB and not in
Fig. 4. Photomicrograph of dark-field images comparing the level of expression of choline acetyltransferase (ChAT; A), VACHT (B), and tyrosine hydroxylase (TH) mRNA (C) in serial 12-μm sections of the CB (A–C) and NG-PG-JG complex (D–F) from 1 animal at postnatal day 4 after a 4-h exposure to hypoxia. Expression of ChAT and VACHT is not above background grains in the CB, whereas large clusters of silver grains seen throughout the CB tissue section represent a high level of expression of TH mRNA (C). Similarly, 12-μm serial sections of NG-PG-JG complex show ChAT (D) mRNA expression above background, whereas VACHT (E) and TH mRNA (F) expression is seen in ganglion cells. Small white dots represent clusters of silver grains on low-power views of the NG-PG-JG complex. Scale bar = 50 μ.m. A–C: high magnification. D–F: low magnification.

Fig. 5. Photomicrograph of bright-field images showing expression of VACHT immunoreactivity (IR) in the CB at postnatal (PN) 4 (A and B) and 2 animals at PN 15 (C–F). Microganglion cells on the periphery of the CB were intensely positive for VACHT IR (A–D). Linear pattern of VACHT IR was seen within the CB (arrows) and IR surrounding clusters of smaller cells (●) were seen. A, C, E: low magnification. B, D, F: higher magnifications of insets of A, C, and E, respectively.
type I cells. Immunostaining of entire CBs from multiple animals allowed us to discern single and sometimes multiple microganglion cells within fibers juxtaposed to distinct clusters of type I cells. These microganglia could account for small amounts of ACh, which were detected by HPLC in in vitro preparations of the CB, as reported by Fitzgerald et al. (13). Also, our ISHH confirmed low levels of ChAT mRNA within these cells, suggesting their capacity to synthesize ACh. In addition, our data support the results of Schütz et al. (35), who showed that the expression of ChAT is far less abundant than VACHT mRNAs in the peripheral nervous system, whereas ChAT and VACHT mRNAs and IR are comparable in the CNS.

Multiple mRNA splice variants exist for the ChAT and VACHT genes. Each of these splice variants occurs within the noncoding regions of the genes and do not affect the coding region. Thus the cDNA that we used for in vitro transcription contained the entire coding region for both of the genes. Nevertheless, a new alternative splice variant and subsequent protein product have been identified for ChAT, which appears to be expressed preferentially in the periphery (pChAT). Tooyama and Kimura (36) cloned pChAT from the rat and showed that the gene product lacked exons 6–9 of the ChAT gene. Rabbit antiserum raised against pChAT has been developed using the 41-amino acid polypeptide that spans the splice joint between exons 5 and 10 (36). The expression of pChAT mRNA and protein was preferentially localized to cholinergic neurons in the periphery vs. those in the CNS (30, 31, 36). Thus the low level of mRNA expression that we detected in the NG-PG-JG complex and perhaps the absence of ChAT expression in type I cells could be explained by the existence of an alternative splice variant that our probes could not detect. On the other hand, a splice variant for VACHT that is uniquely expressed in the periphery, to our knowledge, has not been described. Furthermore, VACHT IR is frequently used to detect cholinergic neurons in the periphery and in the CNS (42). The results of this present study agree with those of Schütz et al. (35), who indicated that VACHT IR is not expressed in type I cells within the CB but that VACHT IR is present within microganglion cells and nerve terminals of the CB in newborn and adult rats. We are aware, however, that the possibility exists that a splice variant of the VACHT gene could be expressed in type I cells, which differs from the splice variant for VACHT that is expressed in the microganglion cells juxtaposed to clusters of type I cells and found in the pre- and postganglionic nerve fibers in the SCG and CB, respectively.

We also observed strong VACHT IR in terminal nerve fibers surrounding clusters of type I cells, which are most likely the postganglionic parasympathetic fibers previously shown by detailed morphological studies performed by McDonald (28) and Wang et al. (39) in the rat CB. Wang et al. also described ganglion cells on the periphery of the rat CB, which coexpress IR for nitric oxide synthase and ChAT; however, IR for ChAT and nitric oxide synthase was not detected in type I cells. Species differences, however, may exist. In earlier studies, Wang et al. (40, 41) reported the presence of ChAT IR localized to type I cells in the carotid bodies of the cat and rabbit. Alternatively, another source of ACh in the CB of the cat could be microganglion cells, since autonomic ganglion cells in the cat CB have also been described by De Castro et al. (see Ref. 28). Our data suggest that microganglion cells in the rat are likely to be a rich source of ACh in the CB. It seems plausible that microganglion cells and cholinergic fibers release ACh in response to hypoxia, which could account for the increase in ACh detected in the perfusate of CBs exposed to hypoxia in vitro (13).

Although the expression of VACHT in peripheral arterial chemoreceptors does not appear to be transcriptionally regulated by subacute (4 h) hypoxic exposure, expression in NG-PG-JG complex does appear to be regulated by development. Similar to the observations of others (35), we found a significant reduction in VACHT mRNA expression in the NG-PG-JG complex during postnatal maturation. Although our study was not designed to determine the mechanism for this reduction during development, several plausible explanations exist. Bone...
morphic genes (BMPs) are members of the transforming growth factor-β superfamily, which are involved in growth and differentiation (27). BMPs specifically promote survival and phenotypic maturation of neurons in the peripheral nervous system and CNS (33, 38). VACHT and ChAT are both transcriptionally upregulated by BMP-9, an important inducer of cholinergic phenotype in the CNS (26). In addition, retinoic acid, cAMP, leukemic inhibitory factor/ciliary neurotrophic factor have all been shown to induce and upregulate VACHT and ChAT mRNAs in cholinergic neurons in culture (3). Either a reduction in BMP-9 and leukemic inhibitory factor/ciliary neurotrophic factor or changes in receptor levels for these ligands on NG-PG-JG ganglion cells during development could result in downregulation of VACHT mRNA expression during maturation. Alternatively, although not addressed by our study, a reduction in the number of cholinergic-expressing cells in the NG-PG-JG complex may occur via programmed cell death during early postnatal maturation in the newborn rat.

In conclusion, the results of this study on the developing rat CB, utilizing semiquantitative ISHH and IH to detect cholinergic traits at the message and protein levels, respectively, indicate that autonomic ganglion cells and cholinergic nerve terminals innervating the CB are a major source of ACh. Furthermore, under the present experimental conditions, 4 h of hypoxic exposure does not affect the levels of VACHT and ChAT mRNA expression in the CB or in ganglion cells within the CB.
the NG-PG-JG complex. However, these findings do not de-
fectively exclude possible contributions from type I cells on
cholinergic transmission in the peripheral arterial chemorecep-
tors in other species (10, 12) or dissociated carotid bodies in
culture (32).

ACKNOWLEDGMENTS

The authors thank Dr. Musa A. Haxhiu for critically reviewing the manu-
script, providing suggestions, and unconditional support. We also thank Dr.
Clarke G. Tankersley for insightful recommendations.

GRANTS

This study was supported by National Institutes of Health Grants RO1 DA-
13940 (to E. B. Gauda and G. L. McLemore) and U54 NS-39407 (to S. M.
Johnson).

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